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TITLE: The Thoc1 Ribonucleoprotein as a Novel Biomarker for Prostate Cancer Treatment Assignment

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Active surveillance (AS) is an option for men with low risk prostate cancer in order to reduce over treatment, but few men choose it because current prognostic indicators are imperfect. The objectives of this research are to test whether pThoc1 can improve the assignment of prostate cancer patients to therapy. We have made significant progress on the goals articulated in the Statement of Work. IRB/HRPO approval has been obtained for construction and use of new TMAs (PI Mohler and Goodrich). The TMAs from PCaP have been obtained (PI Mohler and Goodrich). Pathology analysis of 1146 patient specimens is complete and construction of TMAs is complete (PI Mohler). Optimization of TMA staining is complete and staining of all TMAs initiated, with staining of some TMAs complete (PI Goodrich). IRB/HRPO approval for active surveillance specimens has been obtained (PI Mohler, Goodrich). Enrollment of prostate cancer patients on active surveillance is ongoing (PI Mohler). Developing ELISA assays for measuring pThoc1 and pThoc1 autoantibodies is complete (PI Goodrich). IRB/HRPO approval for serum samples has been obtained (PI Mohler, Goodrich). Analysis of serum samples from human prostate cancer patients has been initiated (PI Goodrich). Year 3 of the grant will finish data acquisition and data analysis. Over treatment complicates the clinical management of prostate cancer. Improving the ability to distinguish aggressive from indolent disease is recognized as an unmet need by the 2013 PCRP Overarching Challenges. Identifying pThoc1 as a biomarker that can help meet this need will have					
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## Table of Contents

	<u>Page</u>
<b>1. Introduction.....</b>	<b>4</b>
<b>2. Keywords.....</b>	<b>4</b>
<b>3. Accomplishments.....</b>	<b>4</b>
<b>4. Impact.....</b>	<b>5</b>
<b>5. Changes/Problems.....</b>	<b>5</b>
<b>6. Products.....</b>	<b>5</b>
<b>7. Participants &amp; Other Collaborating Organizations.....</b>	<b>5</b>
<b>8. Special Reporting Requirements.....</b>	<b>5</b>
<b>9. Appendices.....</b>	<b>6</b>
<b>10. References.....</b>	<b>8</b>

## **1. Introduction**

Active surveillance (AS) has been proposed as an option for men with low risk prostate cancer in order to reduce over treatment. Only a fraction of eligible men choose AS, however, because current prognostic indicators are imperfect. Biomarkers that improve upon PSA levels, clinical stage and Gleason score to distinguish between prostate cancers that can be observed safely from those that require immediate treatment could help “right size” recommended treatment. The objectives of this proposal are to test whether pThoc1 can improve the assignment of prostate cancer patients to therapy, to test whether pThoc1 correlates with observed racial disparities in prostate cancer mortality, to determine whether pThoc1 can identify active surveillance patients whose prostate cancer will progress, and to develop methods to quantitate pThoc1 or pThoc1 autoantibody in serum. The general study design is to assay pThoc1 in independent cohorts of clinically annotated prostate cancer biospecimens for which clinical and follow up data is available using previously developed antibody reagents and immunostaining methods. Over treatment is a critical issue complicating the clinical management of prostate cancer. Improving the ability to distinguish aggressive from indolent disease in men newly diagnosed with prostate cancer is recognized as an unmet need by the 2013 PCRP Overarching Challenges. Identifying pThoc1 as a biomarker that can help meet this need will have significant impact.

## **2. Keywords**

Prostate cancer, biomarker, active surveillance, prognostic indicator, tissue microarray, immunostaining, ribonucleoprotein

## **3. Accomplishments**

There are three major goals for the proposed work: 1) Characterize pThoc1 levels in independent cohorts of human prostate cancer radical prostatectomy specimens. 2) Characterize pThoc1 levels in a cohort of human prostate cancer patients on active surveillance. 3) Test whether pThoc1 or autoantibodies against pThoc1 can be detected in the serum of prostate cancer patients.

The major accomplishments achieved during the first two years of funding period of the grant include successfully securing all IRB/HRPO regulatory approvals to conduct the work, pathological assessment of 1146 patient specimens for use in constructing tissue microarrays, developing and optimizing ELISA assays to detect anti-Thoc1 autoantibodies, and validating those assays using a normal human serum. With respect to the approved Statement of Work, the following objectives were accomplished: Construction of new prostate cancer TMAs (PI Mohler and Goodrich) of 1146 patient specimens is complete. The TMAs from PCaP have been obtained (PI Mohler and Goodrich) and staining of TMAs for Thoc1 protein (figure 1) is complete (PI Goodrich). Pathological scoring of the TMAs is underway (PI Goodrich and PI Mohler). Enrollment of prostate cancer patients on active surveillance is ongoing (PI Mohler). Thoc1 immunostaining of prostate biopsy tissue of patients from PCaP cohort who are candidates for active surveillance is complete (figure 3). Analysis of serum samples for Thoc1 autoantibodies is underway.

A three gene panel constituting PMP22, CDKN1A and FGFR1 has been shown to stratify low gleason score prostate cancers into indolent and aggressive cancer (Irshad et al., 2013). This panel of genes has been shown to have increased expression in low Gleason score prostate cancers that are likely to remain indolent compared to those which will progress and become aggressive prostate cancer. Thoc1 protein on the other hand is highly expressed in those low Gleason score prostate cancers that are likely to progress to aggressive prostate cancer (Chinnam et al., 2014). Hence, Thoc1 expression in low Gleason score prostate cancer is complimentary to that of the panel of PMP22, CDKN1A and FGFR1. Addition of this complimentary panel of genes to our analysis will help us robustly determine if Thoc1 can stratify low Gleason score prostate cancers into indolent and aggressive prostate cancer. Immunostaining for PMP22, one of the complimentary antigen to Thoc1, has been optimized (PI Goodrich). A set of TMAs made from 700 patients available at RPCI has been immunostained for PMP22 (figure 3).

ELISA assays for measuring pThoc1 and pThoc1 autoantibodies have been successfully developed (PI Goodrich). A sandwich ELISA assay has been developed for detecting Thoc1 protein in serum samples. Recombinant Thoc1 protein was produced in E.coli and purified by affinity chromatography. This control

protein was used to develop the sandwich ELISA assay, and a standard curve using the assay is shown in figure 4. An indirect ELISA assay was developed for detecting autoantibodies against Thoc1 protein in serum samples. However, due to high background signal from anti-human IgG secondary antibodies with human serum sample, a competitive ELISA assay has been developed for detecting Thoc1 autoantibodies. Purified anti-Thoc1 mouse monoclonal antibody was used as control antibody with biotinylated anti-Thoc1 monoclonal antibody for generating a competition standard curve (figure 5). The assay was validated by pre-adsorbing Thoc1 autoantibodies using GST-Thoc1 protein (Table 1).

IRB/HRPO approval for human serum samples has been obtained (PI Mohler, Goodrich). Task 1, specific aim 1 is complete. Task 1, specific aim2 is complete. Task 1, specific aim 3 is complete. Task 2, specific aim 3 is ongoing. Overall, optimization, validation, and regulatory approval work has been completed and data gathering for all specific aims is ongoing.

The grant does not support training and professional development, so there is nothing to report.

The work is at a preliminary stage, so results have yet to be disseminated through publication or presentations.

#### **4. Impact**

What was the impact on the development of the principal discipline(s) of the project? ELISA assays were developed for measuring pThoc1 or pThoc1 autoantibodies in serum. Such assays are not currently available, so these assays will permit measurement of this ribonucleoprotein and autoantibodies directed against it. By correlating these measurements against clinical data, we will determine whether pThoc1 or pThoc1 autoantibodies are a useful serum biomarker for prostate cancer. If so, the development of these assays will impact the diagnosis of prostate cancer and possibly other cancers.

If pThoc1 or pThoc1 autoantibodies prove to be a useful serum biomarker for prostate cancer, the work may impact development of diagnostic/prognostic products based on pThoc1.

The presence of the THO ribonucleoprotein complex, or antibodies directed against it, have not been measured in serum or other bodily fluids, either in humans or animal models. These measurements may impact understanding of ribonucleoproteins in general and their ability to elicit autoantibody responses.

#### **5. Changes/Problems**

Nothing to report.

#### **6. Products**

As the work is still at a preliminary stage, it has not yet been disseminated through publications, conference papers, website(s), or presentations. No inventions, patent applications, and/or licenses have been produced.

ELISA assays were developed for measuring pThoc1 or pThoc1 autoantibodies in serum. If utility of these assays as serum biomarkers of prostate cancer aggressiveness is established, results will be shared through journal publications and meeting presentations.

#### **7. Participants & Other Collaborating Organizations**

David W. Goodrich, Ph.D. (Initiating PI): No change.

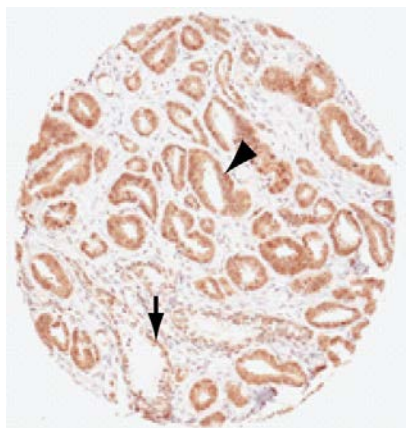
Meenalakshmi Chinnam, Ph.D. (postdoctoral fellow): No change.

There has been no change in active other support for the PI or postdoctoral fellow since the original grant submission. No other organizations are involved in the research.

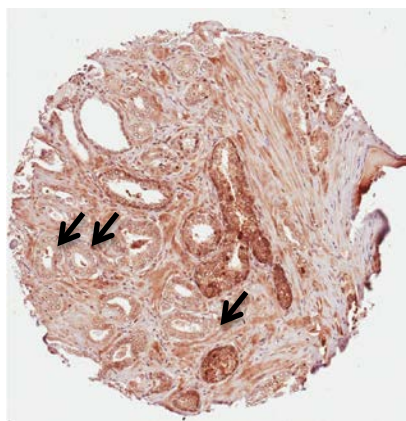
#### **8. Special Reporting Requirements**

This grant funds a Synergistic Idea Development Award in collaboration with Dr. James Mohler (Partnering PI, Roswell Park Cancer Institute). Dr. Mohler will be submitting an independent annual report describing his aspect of the work.

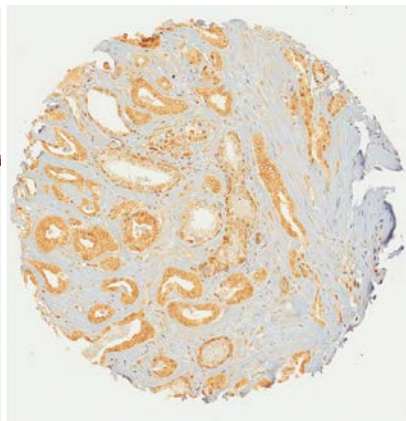
## 9. Appendices



**Fig 1. Thoc1 immunostaining of prostate cancer tissue from PCaP TMA.** TMAs from PCaP project were obtained and immunostained for Thoc1 protein using our optimized protocol. Increased Thoc1 expression is observed in tumor ducts (arrowhead) compared to cells in an adjacent benign duct (arrow).

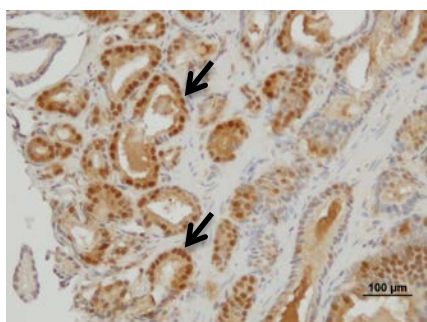


PMP22



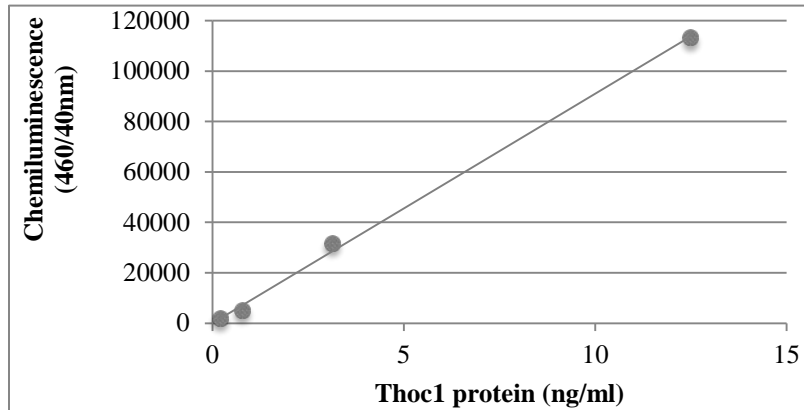
Thoc1

**Fig 2: PMP22 immunostaining of prostate cancer tissue from RPCI TMA.** A set of prostate cancer TMAs from RPCI cohort was immunostained for PMP22 protein (shown on left). TMAs from the same cohort were previously analyzed for Thoc1 protein (shown on right). Note decreased PMP22 staining in cancer ducts (arrows) whereas the same cancer ducts on adjacent TMA show increased

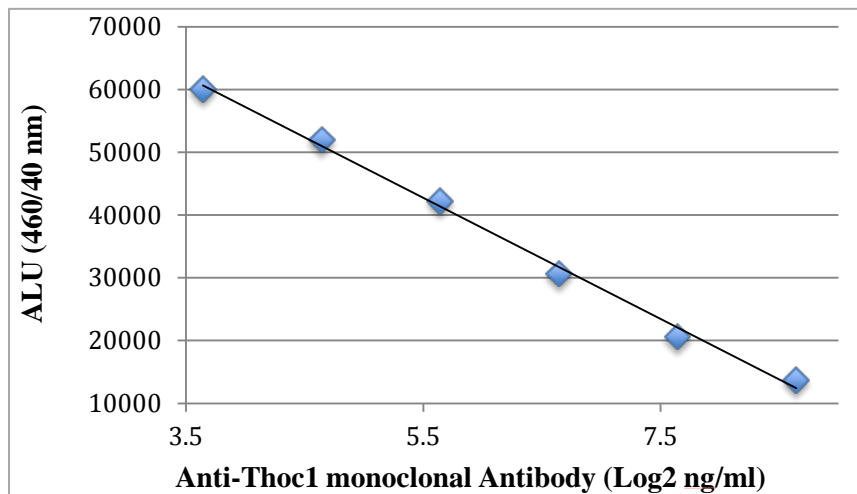


Thoc1

**Fig 3: Thoc1 immunostaining of prostate biopsy tissue from PCaP.** Biopsy specimen of patients in PCaP cohort who qualified for active surveillance were immunostained for Thoc1 protein. Increased Thoc1 expression is observed in atypical cells of cancer ducts (arrows) compared to benign. Also, the staining pattern is heterogenous.



**Fig 4: Measurement of Thoc1 protein by sandwich ELISA.** A range of diluted, purified Thoc1 control protein (0.2-12.5 ng/ml) was assayed by ELISA and the chemiluminescence (460/40nm) recorded after subtracting assay blank. The lowest amount of Thoc1 protein detectable is 200 pg/ml or 10 pg/well.



**Fig 5: Measurement of anti-Thoc1 antibody by competitive ELISA assay.** A competition standard curve was generated from a dilution series (12.5-400 ng/ml) of purified anti-Thoc1 control antibody in competition with anti-Thoc1 biotinylated antibody.

Serial dilution of serum	Thoc1 antibody concentration of normal serum sample (ng/ml)	Thoc1 antibody concentration in normal serum pre-adsorbed with GST-Thoc1 protein (ng/ml)	Thoc1 antibody concentration in normal serum pre-adsorbed with GST protein (ng/ml)
1:10	35.09	2.74	27.55
1:20	5.66	0.91	10.70
1:40	1.88	0.01	4.35
1:80	1.03	0.00	3.22
1:80, spiked with 50 ng/ml control anti-Thoc1 antibody	53.13	0.01	71.93

**Table 1: Validation of competitive ELISA assay for detecting anti-Thoc1 antibody.** Normal human serum sample was divided into 3 parts. One part was pre-adsorbed with GST-Thoc1 protein, second part was pre-adsorbed with GST protein and the third part was not pre-adsorbed. The samples were then analyzed by competitive ELISA assay by serially diluting the serum samples. GST-Thoc1 pre-adsorption of samples depleted Thoc1 autoantibodies as expected whereas pre-adsorption with GST protein alone altered levels of Thoc1 autoantibody minimally. Normal human serum sample diluted 1:80 was spiked with 50 ng/ml control anti-Thoc1 antibody to test the efficiency of pre-adsorption. As shown in the table, the control anti-Thoc1 antibody was efficiently pre-adsorbed in GST-Thoc1 protein and not by GST protein, thus also demonstrating specificity of pre-adsorption.

## 10. References:

Irshad S, Bansal M, Castillo-Martin M, Zheng T, Aytes A, Wenske S, Le Magnen C, Guarnieri P, Sumazin P, Benson MC, Shen MM, Califano A, Abate-Shen C. A molecular signature predictive of indolent prostate cancer. *Sci Transl Med*. 2013 Sep 11;5(202):202ra122.

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